



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No.: P-576 (TI-0022)
Inventors: Huber et al.
Serial No.: 09/770,410
Filing Date: January 25, 2001
Examiner: Therkorn, Ernest G.
Customer No.: 26259
Group App Unit: 1723
Confirmation No.: 6186
Title: Method and Apparatus for Separating
Polynucleotides Using Monolithic
Capillary Columns

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Date of Deposit: March 6, 2006

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By Jane Massey Licati
Typed Name: Jane Massey Licati, Reg. No. 32,257

Commissioner for Patents
Mail Stop AF
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

DECLARATION UNDER RULE 9 1.131

1. Andreas Premstaller, hereby declare that:

2. I am a co-inventor, together with Christian Huber
and Herbert Oberacher, in U.S. Patent Application Serial No.
09/770,410 filed June 7, 2000 and am most familiar with the
subject matter of this application and the research effort which
lead to the discovery of the instant invention. All the work
described in the following paragraph occurred at the Institute of

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Analytical Chemistry and Radiochemistry in Innsbruck, Austria, a recognized WTO member country since January 1, 1995.

2. I have reviewed Gusev et al. ((September 1999) *J. Chromatography* 855:273-293) and find that this reference describes a porous monolithic packing prepared with polystyrene-divinylbenzene support which is covalently attached to a fused silica capillary inner wall treated with a coupling agent trimethoxysilyl propyl methacrylate to provide anchoring sites for grafting of the polymer to the silica surface. The median pore radius for a monolithic sample prepared with ethanol is, as estimated by Gusev, about 5 micrometers.

3. Our invention referenced above, teaches a device for separating a mixture of polynucleotides by ion pair-reversed phase-high performance liquid chromatography. The device comprises a polymeric monolith having non-polar chromatographic surfaces. The monolith comprises an underivatized poly-(styrene/divinylbenzene) matrix and is contained within a tube having an inner diameter in the range of 1 to 1000 micrometers.

4. Laboratory protocol notebooks regarding experiments related to this invention were kept by me as a Ph.D. student under the direction of Christian Huber.

5. I worked in Dr. Huber's laboratory during 1998 and 1999.

6. According to laboratory protocol notebooks submitted herewith, the first synthesis of PS/DVB monolith using decanol and tetrahydrofuran as porogens was performed on August 6, 1996. We then succeeded in a first separation of proteins (lysosome from beta-lactoglobulin B) in a PS/DVB monolithic column on August 25, 1998. See, e.g., the chromatograph at the bottom right-hand corner of the fourth laboratory notebook page. The first successful separation of oligonucleotides on a PS/DVB

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monolith synthesized with decanol/THF as porogens was February 10, 1999.

7. We were able to fully practice our invention described in the above-referenced patent application prior to the date of the publication of the Gusev paper. A copy of the relevant laboratory notebook pages hereby accompanies my declaration.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

24.02.2006

Date



Andreas Premstaller, Ph.D.

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6.8.98

Chloridkopolymer mit THF

C₁₂O₄H₂₂
mischelöslich

Nr.	Datum	Kapillare ID/OD (µm)	Polymerisationsmischung					Temperatur [°C]
			Styrol (ml)	DVB (ml)	AIBN (g)	C ₁₂ OH (ml)	THF (ml)	
M11_1	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	3.00	0.00	70, TS
M11_2	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	2.90	0.10	70, TS
M11_3	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	2.80	0.20	70, TS
M11_4	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	2.70	0.30	70, TS
M11_5	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	2.60	0.40	70, TS

THF sollte kommen, in der Kette ein Molekül stehen als Toluol
THF distilliert, da mit Rückhalteeigenschaft (Theor.) stabilisiert

Ausgangsmaterial: VS 38.98 Tocken
THF dest.

Zeit: 6.8.98 100h
Zeit: 7.8.98 120h

T = 70°C

T = 70°C

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M11_1 16 cm			M11_2 15 cm		
Fluß (µl/min)	Gegendruck (bar)	(bar/cm)	Fluß (µl/min)	Gegendruck (bar)	(bar/cm)
5	1	0.06	10	1	0.07
10	1	0.08	25	4	0.27
25	4	0.25	50	8	0.53
50	7	0.44	100	14	0.93
100	14	0.58	150	20	1.33
150	21	1.31	200	25	1.67
200	28	1.75	k (bar cm ³ µl ⁻¹ min ⁻¹) 0.008303		
k (bar cm ³ µl ⁻¹ min ⁻¹) 0.008712					

M11_3 15 cm			M11_4 16 cm		
Fluß (µl/min)	Gegendruck (bar)	(bar/cm)	Fluß (µl/min)	Gegendruck (bar)	(bar/cm)
10	1	0.07	5	3	0.56
25	3	0.20	10	14	0.88
50	6	0.40	25	32	2.00
100	11	0.73	50	68	4.25
150	14	0.93	100	125	7.58
200	19	1.27	150	150	11.25
k (bar cm ³ µl ⁻¹ min ⁻¹) 0.008114			k (bar cm ³ µl ⁻¹ min ⁻¹) 0.074902		

M11_5 16 cm 200bar Gegendruck			Ansat THF		Stärkung
Fluß (µl/min)	Gegendruck (bar)	(bar/cm)	[% Porogen]		k (bar cm ³ µl ⁻¹ min ⁻¹)
1	21	1.31	0.0%		0.00871
2	33	2.06	3.3%		0.00830
3	49	3.06	6.7%		0.00811
4	63	3.94	10.0%		0.07460
5	77	4.81	13.3%		0.98319
7	92	5.75			
10	170	10.63			
12	184	11.50			
k (bar cm ³ µl ⁻¹ min ⁻¹) 0.983149					

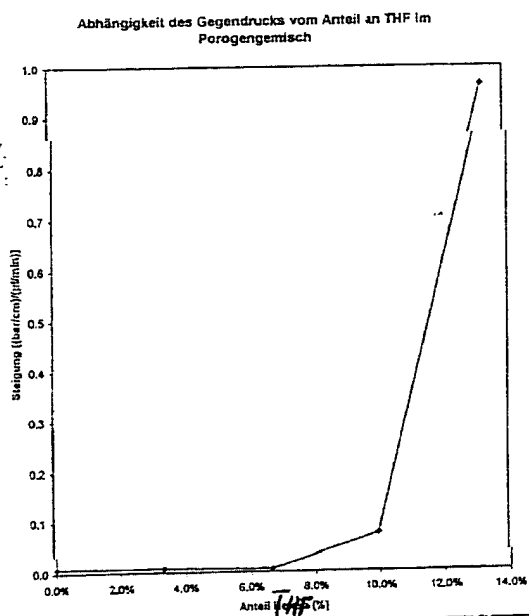
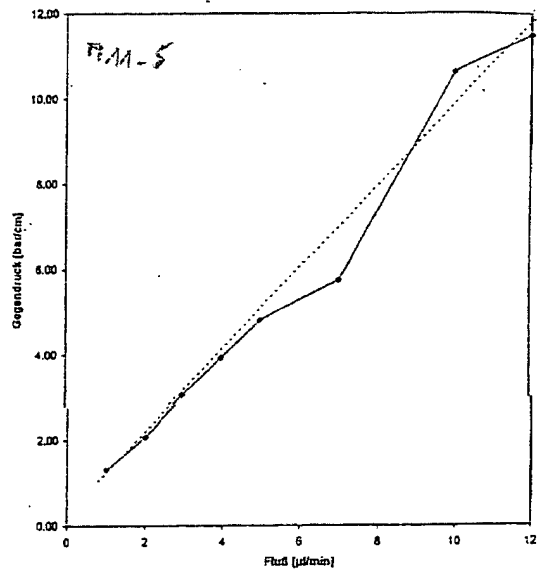
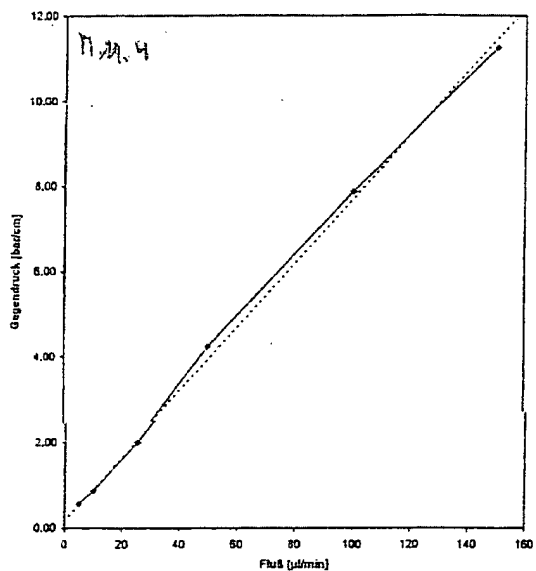
Ultrahoch: 55 Stufen: 450 cm 1 Ter → 8 µm
M11_1 keine Pore ~ 3 µm
sd porös.

M11_2 große Pore, nicht gleichmäßig
M11_3 große Pore, kleine Kanäle (µm - groß)

M11_4 sd dicht, keine Pore, por, da nur
gute zu sehen

M11_5 keine Ultrastrukturen zu erkennen.

el



es möchte sein, dass MA-4 und MA-5 genau stehen.
 für MA-4 und MA-5.

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25.08.98

M 11.5 ml 6.8.98

1ml 3µl 240 bar / 5 µl/min

Fig. AP80875 S170
GYNES-500

SYNAP. 130 µl/min → Split → 4.6 µl/min
2 ml 1000 / 10 µl
2 ml 30 sec 4 µl/min

Equilibrium:

(A) H₂O 0.1% TFA

(B) ACN 0.1% TFA

50% A 14.50 -

Elution - T-Hold 10µl 2 ml 15 sec T-Hold

10µl 2 ml 15 sec 10 / 2.75 4.44 µl/min

Baseline:

Thiokontakt 0.05% H₂O 50% ACN, 0.1% TFA

p ≈ 200 bar

100% H₂O, 0.1% TFA: Proteine zeigen kein Peak → kleine Kapillare?

Thiokontakt near co. 1.50 min

50% ACN, 0.1% TFA: Proteine gleichzeitig mit Thiokontakt: keine Reaktion
RTBA

27.8.98

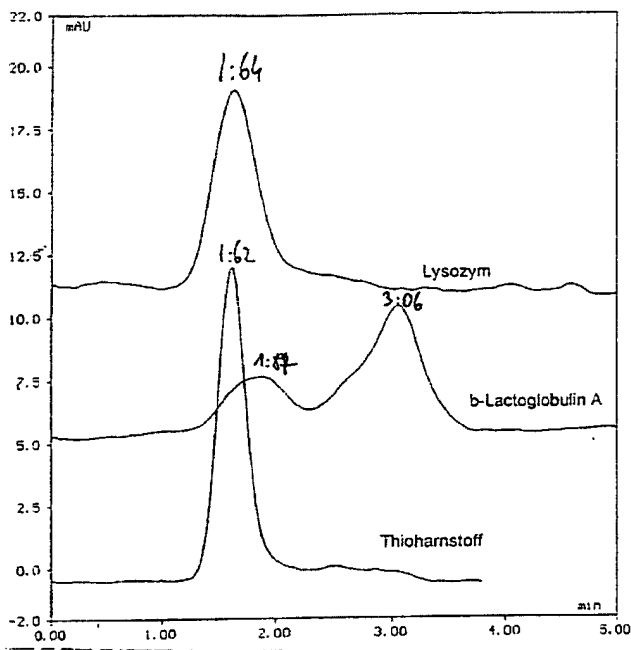
50% ACN, 0.1% TFA: Protein quickly eluted, eluted as Peak.

LAC A.

LYS keine Reaktion

Now 40% ACN

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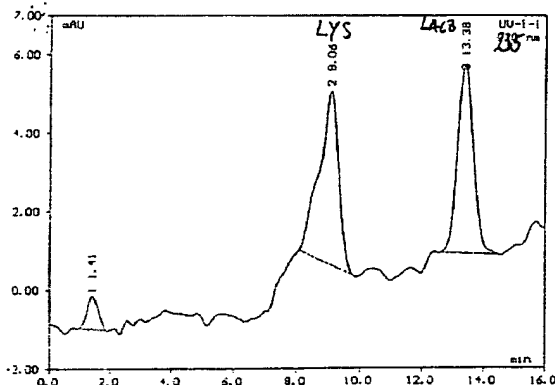
50% ACN, 0.1% TFA
 of Lysozym Protein
 Retention on LacA is 50% ACN

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GP: Integration SYSL - C:\A980825.SMP Page 2
 95DVB 100x0.32mm, C120H/TNF-Perogen, M11.5 38084 1998-08-27/20:13
 LysLacB 1mg/ml UV-1-1 1998-08-27
 Modified: 820/33-6.15 minACN/0.15TFA, 4.5/130ul/min, 25°C SynchroSoft V5.50
 Exp. No/Pos: 35/1 Control: Standard: Inject: 20.0 ul
 Sample Type: Integration Signals: AND11.SIG DIL. Fact.: 1.00000
 Acquisition: 1998-08-27/19:53 Report: Weight: 1.00000
 Method: DEFAULT.LMT P-Table:

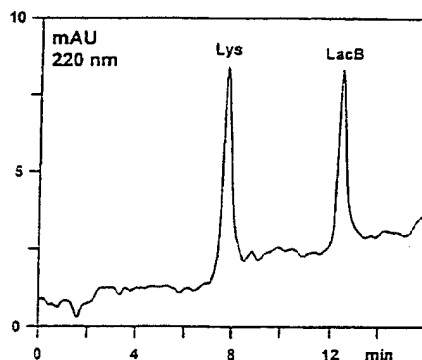
Capillary Protein therapy:
 Lys, LacB of 1mg/ml, 20ul 1/2.

30-60% ACN / 15min, 0.1% TFA
 4.5 / 130ul/min
 215 nm



No.	Ret. Time	Type	Area	Height	Half Width	Base Width	Plates
1	1.414	MS	3.551e+1	0.84	0.415	0.720	64
2	9.060	MS	3.170e+0	4.46	0.627	0.972	1156
3	13.379	MS	2.320e+0	4.82	0.567	0.388	1080
---	---	---	5.443e+0	10.12	---	---	---

A980825-36



Separation of proteins in a monolithic capillary column

Column, PS-DVB (monolith, 100 x 0.32 mm); chromatographic conditions, mobile phase, (A) H₂O, 0.1% TFA, (B) ACN, 0.1% TFA, linear gradient, 30-60% B in 15 min; flow rate, 4.5 µl min⁻¹; temperature, 25 °C; detection, UV, 220 nm; sample, lysozyme, β-lactoglobuline B, 20 ng each.

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Hand line Trennung von Oligonucleotiden in Abwischen 1713.5

1713-5

$\lambda = 87 \text{ nm}$, $\text{id} = 200 \mu\text{m}$

Eluent: A: 50mM TEAA pH 6.8

B: 50mM TEAA 20% ACN pH 6.8

Temperatur: 50°C

Spaltkopf: TSP075375, 6 cm Fluss 120 / 3.3 / 11 min / 94 bar

Job: A990209.S171

Trennung von dT_8 , dT_{16}
 Testzeit mit Gradient 0-100% B/10min. 0.11 min
 = 6.65

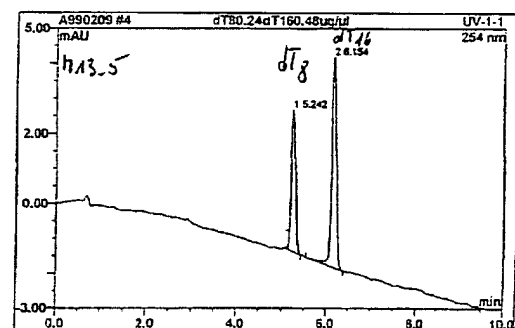
Trennung mit dT_{12-18}

verschiedene Gradienten versucht.

gute Trennung: 30-50% B/10min

6-10% ACN/10min

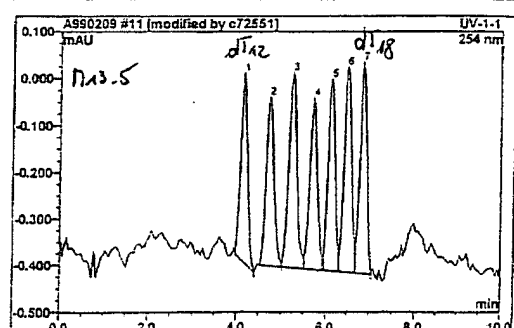
4 dT80.24dT160.48ug/dl
 0-100%B/10min; A: 50mM TEAA pH 6.8; B: 50mM TEAA 20% ACN pH 6.8; 120/3.3 μl/min; D: 2min; 50°C
 Sample Name: dT80.24dT160.48ug/dl Injection Volume: 20.0 μl
 Control Program: Channel: UV-1-1
 Quantif. Method: OLIGO1 Recording Time: 09.02.99 19:00



No.	Ret. Time min	Area mAU*min	Height mAU	Half Width min	Plates (EP)	Asymmetry (AIA)
1	1.5242	0.450	4.053	0.105	13593	1.303
2	2.8154	0.744	8.028	0.111	10926	1.331
Total:		1.194	10.081			

Operator: c72551 Timebase: A990209 Sequence: A990209 Page 11-1
 10.2.1999 2:34 PM

11 dT12-18 0.25ug/dl
 30-50%B/10min; A: 50mM TEAA pH 6.8; B: 50mM TEAA 20% ACN pH 6.8; 120/3.3 μl/min; D: 2min; 50°C
 Sample Name: dT12-18 0.25ug/dl Injection Volume: 20.0 μl
 Control Program: Channel: UV-1-1
 Quantif. Method: OLIGO1 Recording Time: 09.02.99 21:29



No.	Ret. Time min	Area mAU*min	Height mAU	Half Width min	Plates (EP)	Asymmetry (AIA)
1	4.156	0.075	0.405	0.175	3142	1.050
2	4.707	0.071	0.384	0.178	2860	1.551
3	5.224	0.088	0.420	0.182	4101	1.248
4	5.709	0.073	0.370	0.180	5552	1.084
5	6.122	0.082	0.412	0.189	5813	n.a.
6	6.483	0.086	0.441	0.182	7042	n.a.
7	6.835	0.082	0.454	0.171	8586	n.a.
Total:		0.556	2.988			